Advances in Bioscience and Biotechnology, 2024, 15, 637-654
https://www.scirp.org/journal/abb
ISSN Online: 2156-8502
ISSN Print: 2156-8456

Microbial Diversity and Key Metabolic Pathways in Lignite-Promoted Anaerobic Fermentation with Residual Sludge

Yawei Zhang[1], Hongyu Guo[1,2], Daping Xia[2,3*], Shufeng Zhao[1], Ze Deng[4*], Dan Huang[5], Bing Li[1], Yinchuan Li[3]

[1]School of Energy Science and Engineering, Henan Polytechnic University, Jiaozuo, China
[2]Collaborative Innovation Center of Coalbed Methane and Shale Gas for Central Plains Economic Region, Jiaozuo, China
[3]Institute of Resources and Environment, Henan Polytechnic University, Jiaozuo, China
[4]Research Institute of Petroleum Exploration & Development, Beijing, China
[5]Yellow River Conservancy Technical Institute, Kaifeng, China
Email: *xiadp22@hpu.edu.cn, *3663521061@qq.com

How to cite this paper: Zhang, Y.W., Guo, H.Y., Xia, D.P., Zhao, S.F., Deng, Z., Huang, D., Li, B. and Li, Y.C. (2024) Microbial Diversity and Key Metabolic Pathways in Lignite-Promoted Anaerobic Fermentation with Residual Sludge. *Advances in Bioscience and Biotechnology*, **15**, 637-654.
https://doi.org/10.4236/abb.2024.1511040

Received: November 12, 2024
Accepted: November 24, 2024
Published: November 27, 2024

Abstract

To enhance methane production efficiency in lignite anaerobic digestion and explore new ways for residual sludge utilization, this study employed the co-fermentation of lignite and residual sludge for biomethane conversion. The bacterial colony structure, metabolic pathways, and interactions between residual sludge and lignite in anaerobic methanogenic fermentation with different mass ratios were analyzed using macrogenomics sequencing. This study aimed to explore the mechanisms involved in the co-anaerobic fermentation of lignite and residual sludge. The results indicated that the addition of sludge enhanced the metabolic pathways in hydrolysis acidification, hydrogen-acetic acid production, and methanation phases. Notably, the enhancement of acetate- and carbon dioxide-nutrient metabolic pathways was more pronounced, with increased activity observed in related enzymes such as acetic acid kinase (k00925) and acetyl coenzyme synthetase (K01895). This increased enzymatic activity facilitated the microbial conversion of biomethane. The results of the study indicated that the sludge exhibited a promotional effect on the methane produced through the anaerobic fermentation of lignite, providing valuable insights for lignite and residual sludge resource utilization.

Keywords

Lignite, Residual Sludge, Anaerobic Fermentation, Bacterial Colony Structure, Metabolic Pathway

1. Introduction

Coal, being a primary energy source in China, has garnered attention to the imperative need for the rational use of coal, as well as its conversion and utilization. Furthermore, addressing the environmental issues associated with coal combustion, particularly through clean coal technology, is crucial for enhancing coal efficiency and mitigating environmental pollution [1]. In September 2020, the Chinese government proposed a dual carbon development goal, aiming to achieve peak carbon emissions by 2030 and carbon neutrality by 2060 [2]. Studies have shown the potential conversion of organic matter in coal into methane, facilitated by various functional microorganisms, especially methanogenic bacteria capable of converting methoxyaromatic compounds within coal [3]. However, challenges persist in coal-to-bio-methane conversion, such as low methane accumulation, short gas production cycles, and inherent difficulty in degrading the complex structure of coal [4] [5]. Residual sludge, a by-product of municipal wastewater treatment, poses environmental and health risks due to its complex composition, high water content, and susceptibility to decomposition [6]. Furthermore, with an estimated production of 90 million tonnes (80% water content) by 2025 in China, effective management of residual sludge is crucial [7]. Anaerobic digestion, a widely used sludge resourcing technology, utilizes anaerobic microorganisms to convert complex organic matter into energy sources such as volatile fatty acids and methane [8] [9].

In recent years, the co-fermentation of different substrates has become a hot research topic in anaerobic fermentation. Notably, the use of lignite in synergistic anaerobic fermentation with kitchen waste, straw, and residual sludge enhances biomethane conversion efficiency, balancing nutrient composition [10]-[12]. Co-fermentation overcomes limitations of single-substrate fermentation, increasing organic matter type, and addressing challenges in the anaerobic fermentation process, such as microflora degradation difficulties. However, there remains a significant research gap concerning the relationship between microbial flora structure, metabolic pathways, and key genes in the anaerobic fermentation of residual sludge and lignite.

Therefore, in this study, three sets of experiments with different ratios of residual sludge co-anaerobic fermentation with lignite were conducted to analyze the stages of degradation and transformation of methane. This involved characterizing the macrogene variability of biomethane conversion, including changes in carbohydrate-active enzyme composition, hydrolytic acidification metabolic pathways, hydrogen and acetic acid production pathways, and differences in biomethane pathways. The study offered insights into the lignite anaerobic fermentation process with added sludge, elucidating the mechanism facilitating methane production. Additionally, this study provides a new reference for the resource utilization of lignite and residual sludge.

2. Materials and Methods

2.1. Sample Collection and Preparation

Lignite samples were obtained from Daliuta Mine in Inner Mongolia (China),

crushed in a pulverizer and sieved through a 100 mesh (0.150 mm) stainless steel sieve [13] [14]. Subsequently, the processed samples were sealed in a ziplock bag for anaerobic fermentation gas production experiments. The residual sludge (secondary sedimentation tank) was taken from a wastewater treatment plant in Jiaozuo, China. The percentage of chemical composition in the residual sludge was characterized by 34% carbohydrates, 12% proteins, and 8% fats, which were also stored for experimentation. According to ISO 17246-2010, the lignite's proximate analysis was performed and the results are shown in **Table 1**.

The bacteria were extracted from the residual sludge of a wastewater treatment plant in Jiaozuo, China, and the bacterial enrichment culture was set up in accordance with a method previously described in the literature [15] [16]. The culture medium was used to enrich the microflora and kept in the laboratory for anaerobic fermentation gas production experiments.

Table 1. Coal quality analysis-related parameters.

Sampling point	Proximate analysis w_t%			Ultimate analysis w_t%				
	M_{ad}	A_{ad}	V_{daf}	C_{daf}	H_{daf}	N_{daf}	O_{daf}	S_{daf}
Daliuta coal mine in Inner Mongolia	7.84	10.87	41.03	80.54	4.75	1.03	2.35	11.14

M, moisture; *A*, ash yield; *V*, volatile matter; *C*, fixed carbon; ad, air-dry basis; daf, dry ash-free basis; C, carbon; H, hydrogen; O, oxygen; N, nitrogen; S, sulfur.

2.2. Instruments and Methods

2.2.1. Experiment on Anaerobic Fermentation of Sludge with Lignite in Different Ratios

The residual sludge, diluted to 1000 mg/L and coal fixed at 100 mg/L was utilized in three sets of biochemical methanogenesis experiments with varying VSS mass ratios of S (sludge): L (coal) of 0:1, 1:1, and 3:1. Conical flasks, N_2 was used instead of oxygen in the bottle to achieve an anaerobic environment, were hermetically sealed and placed in a thermostat incubator (35˚C) anaerobic reactions. Until the end of gas production, the composition changes and gas output were notedonce each day. A gas chromatograph (Agilent 7890GC; Agilent Technologies Inc., Santa Clara, CA, USA) was used to examine the composition of the biogas after it had been collected using a gas collecting bag.

2.2.2. Metagenomic Sequencing Analysis

In this study, Peak gas production samples were collected from reaction groups and metagenomic libraries (2 × 150 bp) were prepared based on the Illumina MiSeq/NovaSeq/HiSeq high-throughput sequencing platform using the Illumina Nexttera DNA XT kit (Illumina, San Diego, CA, USA). Macrogenomic DNA obtained from fermentation samples was extracted and the V3 - V4 hypervariable region of the bacterial 16S rRNA gene was amplified using primers 338F and 806R. Obtain clean sequences by merging, filtering and quality controlling paired-end reads. The paired-end libraries are constructed using the NEB Next® Ultra™ DNA Library Prep

Kit for Illumina®, assembled by splicing with optimized sequences, and then sequenced on the Illumina Genome Analyzer. The resulting genes are annotated for species and function, and categorized. Comparative analysis of samples based on KO data and mapping to KEGG pathway maps was conducted, and MinPat was used to infer the existence and abundance of KEGG pathways. Majorbio BioPharm Technology Co. (Shanghai, China) was the subject of the experiments.

3. Results and Discussion

3.1. Analysis of Gas Yield Results

The results of methane yield experiments with different ratios of sludge and lignite in anaerobic fermentation are shown in **Figure 1(a)-(b)**. The cumulative biogas yield and biomethane yield of the mixed substrate exceeded that of the single lignite. Furthermore, during the anaerobic fermentation methane yield cycle, the gas yield at the daily of incorporating different quantities of sludge exhibited a trend of increasing and then decreasing and essentially halted gas production after 21d. The gas production of the mixed substrate was higher compared with the single lignite. The cumulative methane yield was 87.3 m L/g-VSS for the sludge and lignite mass ratio of 0:1, 123.3 mL/g-VSS for the sludge and lignite ratio of 1:1, and 142.8 mL/g-VSS for the sludge and lignite ratio of 3:1. The methane yield in the 3:1 group was higher compared with the other experimental groups, and the total biogenic gas yield in the 3:1 group reached 1.64 times compared with the 0:1 group. The anaerobic fermentation of the mixed substrate facilitated the biomethane yield. Sludge contains higher quantities of proteins and fatty acids, whereas low-order lignite is characterized by an abundance of side-chain functional groups or free functional groups [17]. These groups are susceptible to chemical bond breaking and reorganization, and concurrently, they serve as readily utilizable substrates for microbial flora transformation, supporting the sustenance of microbial reproduction [5]. Across the three proportioning systems, gas yield efficacy increased with the addition of sludge. Notably, the 3:1 group, featuring a higher percentage of sludge, demonstrated the most favorable

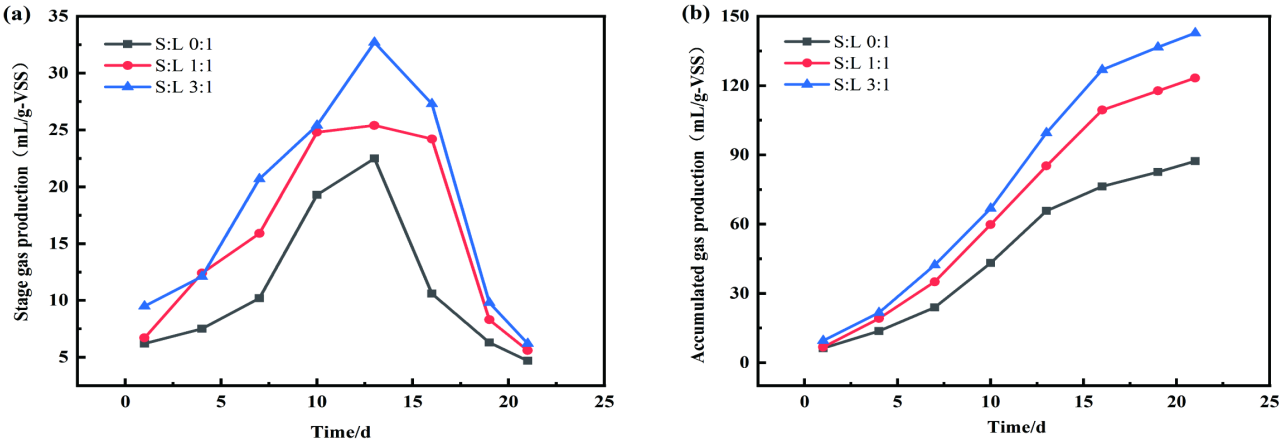

Figure 1. (a) Daily methane yield, (b) Cumulative methane yield.

gas yield effect. In summary, sludge addition facilitated the yield of material gas from lignite anaerobic fermentation, enhancing the biomethane potential of lignite, and facilitating the transformation of organic matter in the anaerobic fermentation substrate.

3.2. Microbial Community Analysis

3.2.1. Microbial Diversity

Table 2 presents the results of alpha diversity analysis at the peak of sludge and lignite ratios of 0:1, 1:1, and 3:1 reactions. ACE and Chao1 indices were commonly used to estimate species richness, while the Shannon and Simpson indices were used to measure species diversity. There was a slight difference in species richness and evenness between the three different ratios of both sludge and lignite at the peak of anaerobic fermentation gas production. The community performance was impacted by the increase in community richness with the augmentation of sludge in the reaction system. However, the distribution of strains was non-uniform, with dominant strains holding a prominent position. Given the gas production effect illustrated in Figure 1, it was evident that greater diversity among dominant flora corresponded to increased participation of microorganisms in the methane conversion process, thereby enhancing the efficiency of biological gas production.

Table 2. Alpha diversity of microorganisms in three different ratios.

Sample	ACE	Chao1	Shannon	Simpson
0:1	310	310	2.654116	0.196735
1:1	260	260	2.550321	0.212442
3:1	561	561	2.743622	0.178226

3.2.2. Microbial Composition and Analysis

To further elucidate the differences in the Microbial of the three different ratios of sludge and lignite at peak gas production, the Microbial categories of the three sets of reactions were analyzed. Figure 2(a) exhibited the dominant microorganisms at the gate level for the three different ration systems. *Bacteroidetes* (43%, 15%, and 13%); *Proteobacteria* (5%, 34%, and 12%); and *Euryarchaeota* (24%, 10%, and e14%) were the main species. *Bacteroidetes* contained a wide range of different hydrolytic, acid-producing, and fermentative bacteria. They played an important role in the degradation of complex organic matter, proteins, lipids, and sugars during hydrolysis and acidification, breaking down complex macromolecules into simpler compounds. These were the dominant functional groups of the anaerobic digestion process [18]-[20] The main role of *Proteobacteria* in wastewater treatment is the removal of organic pollutants, denitrification, and phosphorus removal [21] [22]. *Euryarchaeota* contained a rich diversity of methanogenic archaea. These archaea can use H_2/CO_2, formic acid, methanol, methylamine and acetic acid to produce CO_2, H_2 and methane. The more abundant *Firmicute* phylum is also typical of functional phyla that utilize electron acceptors for anaerobic

metabolism, are involved in hydrogen and acetic acid production, etc. [23] [24]. As shown in **Figure 2(a)**, the relative abundance of *Euryarchaeota* in the sludge–lignite ration ratio of the 3:1 system was 24%. The relative abundance of methanogenic archaea in the 1:1 system was reduced by 4% compared with the 0:1 group, which was reduced by 14%. Furthermore, compared with the 3:1 group, and the abundance of its *Bacteroidetes*, the phylum of bacteria that hydrolyzed and acidified organic substrate was higher than that in the two groups of the ratio system of 0:1 and 1:1. It was observed that the enrichment of hydrolytic flora, such as *Bacteroidetes*, facilitated the degradation of organic macromolecules, providing sufficient substrate for methanogenic archaea [25].

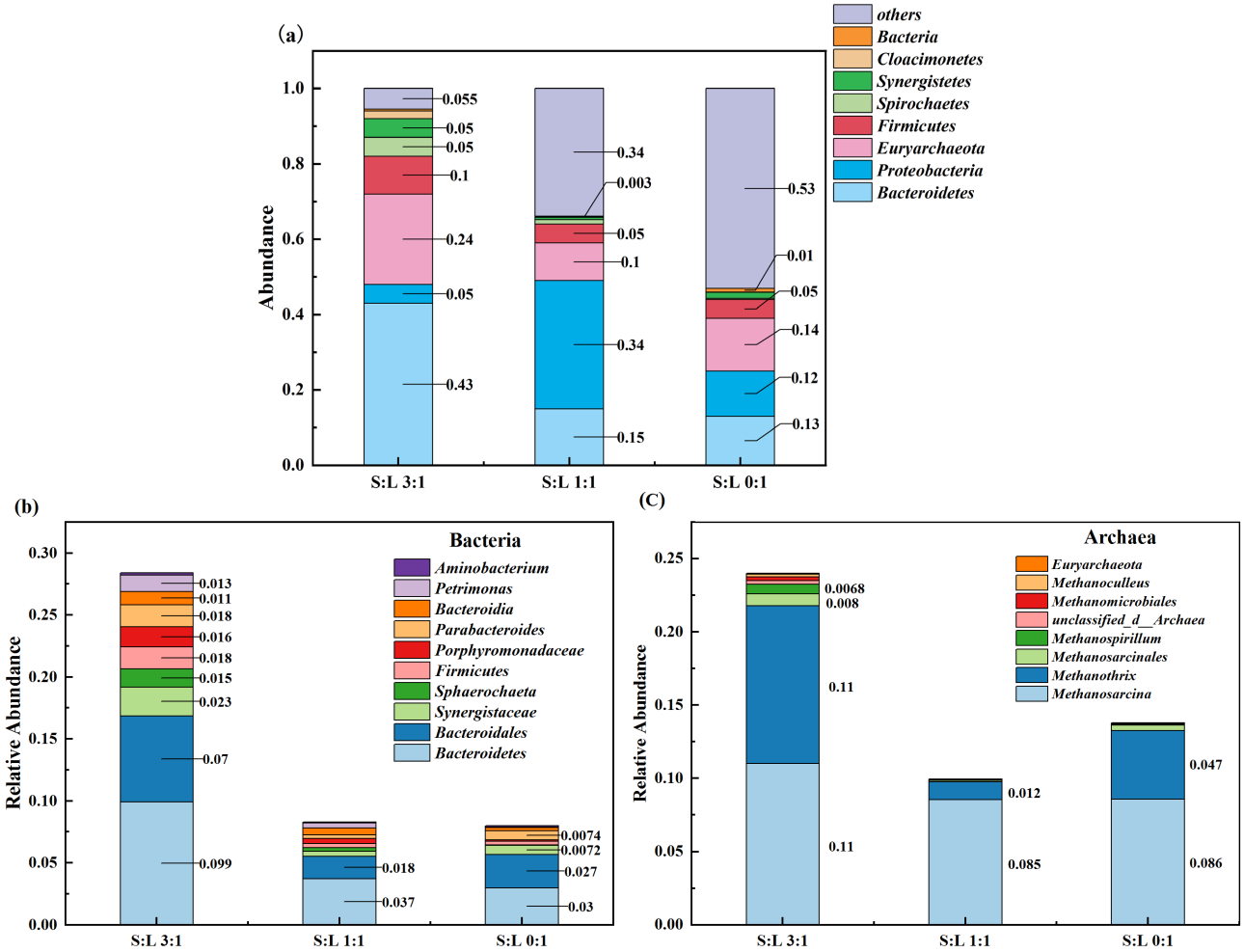

Figure 2. Dominant microorganisms (a) Dominant microorganisms at phylum level, (b) dominant bacteria, (c) dominant archaea.

Figure 2(b) and (c) show the dominant bacteria and archaea at genus levels for each proportioning system. The microorganism community composition was dominated by *Bacteroidetes, Bacteroidales, Sphaerochaeta, and Aminobacterium*. The higher abundance of microorganism at the genus level correlated with the dominant microorganisms exhibited at the phylum level (**Figure 2**). Among these, *Bacteroidetes and Bacteroidales* are both anthropomorphic genera, the former

only degraded propionic acid, while the latter degraded both a variety of fatty acids ranging from C4 to C8, as well as propionic acid [26]. Members of the genus *Sphaerochaeta* are able to hydrolyze a wide range of carbohydrates and organic acids [27] [28]. From the data of the top ten relative abundance of bacterial communities in **Figure 2(b)**, it was observed that among the three different system ratios, the system with 0:1 sludge to lignite ratio contained the lowest *Petrimonas* and *Sphaerochaeta* at 0.09%. Additionally, while the system ratio was 3:1, the contents of these types of characteristic microorganisms increased by 1.3% and 1.5%, respectively [29].

In **Figure 2(c)**, the composition of the archaea among the three different ration systems at the peak of gas production was dominated by *Methanosarcina, Methanothrix, and Methanosarcinales*. The most abundant *Methanosarcina* produced CH_4 by cleaving acetic acid and reducing methyl carbon. Additionally, the hydrogenophilic methane-producing archaea utilized a mixture of CO_2, H_2, and formic acid to produce methane [30]-[32]. *Methanothrix* was the second most abundant archaeon during the peak gas production period. It is a typical acetic acid-nutrient methanogenic archaeon whose energy metabolism is characterized by the catabolism of acetic acid to CH_4 and CO_2 [33]. *Methanothrix* content was 9.8% higher than in the 1:1 and 6.30% higher than in the 0:1 in the sludge-to-lignite ratio 3:1 system.

In summary, the relative abundance of hydrolyzing and acidifying bacteria as well as methanogenic archaeal genera at both the phylum and genus levels, was higher compared with the other two groups in the residual sludge-to-lignite ratio of 3:1 system. This resulted in a higher methane production in the 3:1 group compared with the two outer groups.

3.3. Key Metabolic Stages and Enzyme Activity

3.3.1. Hydrolytic Acidification Process

In anaerobic fermentation, macromolecular organic matter undergoes decomposition into small molecules by extracellular enzymes. The hydrolysis of carbohydrates constitutes a pivotal aspect of this process. Carbohydrate-active enzymes, are functional enzyme systems responsible for the degradation, modification, and synthesis of glycosidic bonds in carbohydrates, serving as the fundamental functional units in the metabolic pathway of saccharides. These enzymes were classified into Glycoside Hydrolases (GHs), GlycosylT-ransferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs), and other CAZymes [34] [35]. Complex carbohydrates and glycocomplexes underwent hydrolysis, yielding small molecules. Glycoside Hydrolases are a category of enzymes that catalyze glycosidic bond hydrolysis in diverse sugar-containing compounds to produce monosaccharides, oligosaccharides or glycoconjugates. GH is frequently employed as a key metric for assessing the hydrolytic capacity of carbohydrates [36] [37]. As presented in **Table 3**, several GH families with higher abundance contained enzymes, cleaving polysaccharides into monosaccharides, such as GH2 (β-Galactosidase), GH20 (β-Glucosidase), and GH57 (α-galactosidase) [39]. The content of GHs in

the 3:1 anaerobic fermentation reactor was higher than that of the corresponding 0:1 and 1:1 GHs. The increase in the abundance of hydrolyzing proteases distinctly characterized the capacity of the colony to hydrolyze macromolecules. The greater the hydrolysis capacity, the smaller molecules were utilized, ultimately enhancing methane production during anaerobic fermentation [39]. Additionally, GH20, GH92, and GH57 contained various GHs such as β-1,6-N-acetylglucosaminidase, α-1,6-mannosidase, β-glucosidase, α-galactosidase, and others, breaking down cell structures and hydrolyzing glycosidic linkages. This played a crucial role in the hydrolysis of sugars and glycoconjugates in organisms [40]. The relative abundance of GH20 and GH92 (**Table 3**) in the reaction group with a 3:1 ratio system was higher compared with the other two groups, facilitating carbohydrate hydrolysis. GH109 was mainly involved in carbohydrate skeleton degradation (higher content in the 3:1 system compared with the other two groups), facilitating the production of volatile fatty acids [41]. The hydrolases contained in GH13 and GH33 hydrolyzed glucosidic bonds and disrupted cellular structure, which were essential in microbial carbohydrate metabolism. The abundance of these two active enzymes in the 3:1 ratio system (**Table 3**) was significantly higher compared with that of the other two groups [42] [43]. In summary, the reaction group with a 3:1 ratio of residual sludge to lignite exhibited a higher abundance of hydrolytic extracellular enzymes released by the bacteria during anaerobic fermentation compared with the other two groups. This condition facilitated the hydrolysis of macromolecular organic matter.

Table 3. The 10 GH clades with the highest expression abundance.

GH[a]	S:L (0:1)	S:L (1:1)	S:L (3:1)
GH2	17,866	13,516	31,544
GH20	6204	8672	21,216
GH92	6032	8148	28,892
GH78	5405	10,320	21,918
GH109	18,042	19,346	19,358
GH29	4862	6644	13,320
GH23	7312	12,486	12,598
GH13	5378	5328	11,002
GH33	8418	12,596	10,652
GH57	5680	9934	10,936

Several different metabolic pathways existed within the anaerobic system, and the metabolic pathways present during acid production determined the overall conversion rate of the anaerobic fermentation [44] [45]. As shown in **Table 4**, the glycolytic metabolic pathway gene abundance expression was the highest in the three different ration reaction groups, followed by the amino acid pathway, and the fatty acid oxidation pathway was the lowest. This shows that the glycolytic

pathway was the most important acid production pathway in anaerobic fermentation. Among the three sets of reactions, the highest abundance of glycolytic genes was found in the sludge-to-lignite ratio of the 3:1 set, followed by the 1:1 set, and the lowest abundance was found in the 0:1 set. This indicated that the higher abundance and activity of acidifying flora in the 3:1 group with higher sludge addition in the three reaction systems facilitated the production of methane in the reaction.

Table 4. Gene abundances corresponding to major metabolic pathways in the acidification stage of hydrolysis.

Metabolic pathways	S:L (3:1)	S:L (1:1)	S:L (0:1)
Glycolysis	261,996	231,706	221,220
Amino acid metabolism	29,620	24,150	20,178
Fatty acid oxidation	18,766	17,386	14,356

Figure 3 shows the main pathways in glycolysis, as well as the key enzyme genes and their abundances for each pathway. In **Figure 3**, the abundance of each key gene in the sludge-to-lignite ratio 3:1 group was generally higher compared with 1:1 and 0:1. The gene abundance of glucokinase (K25026) in the reaction group with a 3:1 ratio was 1.87 and 1.97 times that of 0:1 and 1:1 ratio, respectively. The 6-phosphofructokinase (K00850) abundance was 1.03 and 2.11 times that of 0:1 and 1:1 ratio, respectively. The abundance of expressed genes of phosphoglycerate kinase (K00927) was 1.52 and 1.11 times that of 0:1 and 1:1 ratio, respectively. Thus, the magnitude of activity of certain kinases in glycolysis directly affected the rate and direction of metabolic pathways [46]. The abundance of key enzyme genes associated with acidification in anaerobic fermentation was higher in the reaction group with a 3:1 ratio of residual sludge to lignite. This observation correlated with the distribution pattern of hydrolytic and acidifying flora, as depicted in **Figure 2**, where higher abundance at the phylum and genus levels was evident. Consequently, this enhanced abundance facilitated the acidification reaction.

3.3.2. Acetic Acid Production-Related Pathways and Expression of Related Enzymes

In this study, based on the analysis of the community structure of dominant microorganism at the genus level in **Figure 2(c)**, the acetate-nutrient methanogenic pathway was the main pathway for the methanogenic process of the three mating systems of residual sludge and lignite.

Volatile fatty acids produced during the acidification stage of hydrolysis were further degraded to acetic acid at this stage, where acetyl coenzyme A served as an important precursor for acetic acid production [47]. It was produced primarily from the following two stages: the reduced acetate coenzyme A pathway and the glycolytic pathway. The two acetic acid synthesis pathways are shown in **Figure 4(a)**. In the reductive acetyl coenzyme A pathway, microorganisms utilize H as an electron donor and CO2 as an electron acceptor and building block for

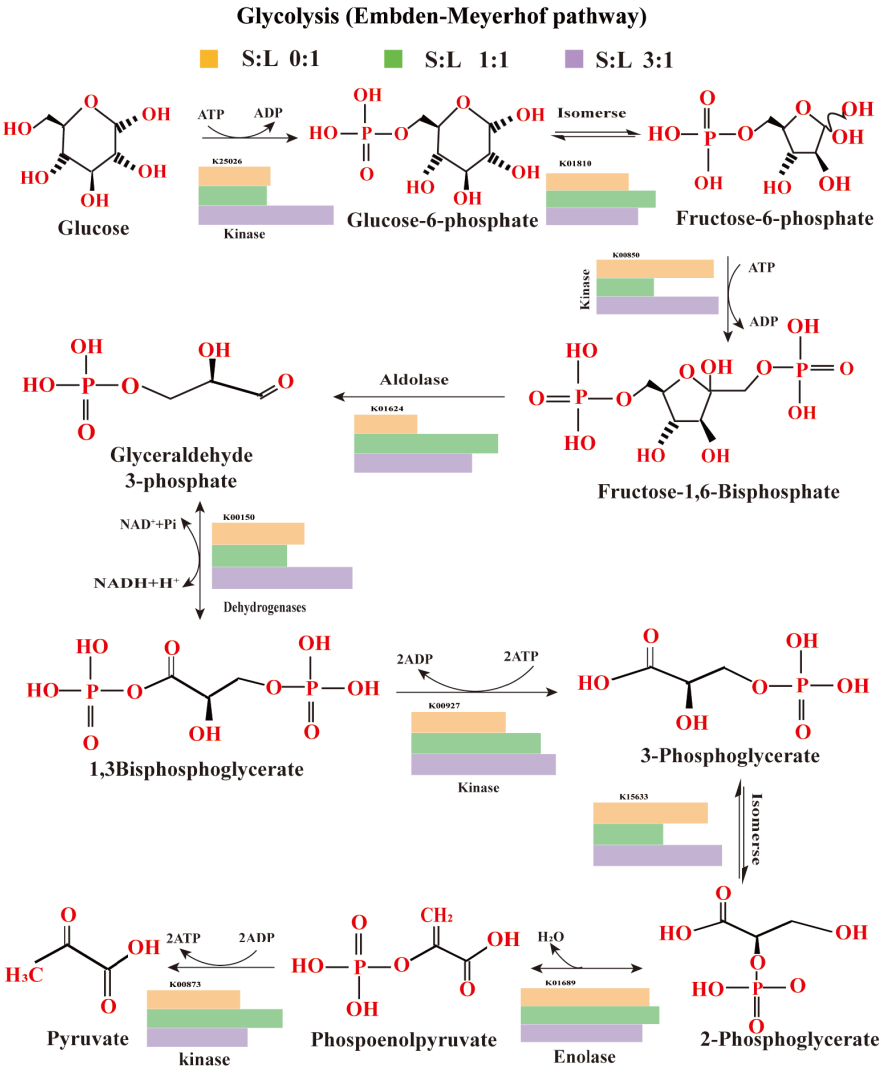

Figure 3. Glycolytic metabolism pathways and abundance of key enzyme genes.

biosynthesis, ultimately leading to the generation of acetyl coenzyme A [48] [49]. Furthermore, carbon monoxide dehydrogenase and acetyl-CoA synthetase play an important role in the reaction to produce acetyl-CoA. As shown in **Figure 4(b)**, the gene abundance of the reaction group with a 3:1 ratio of residual sludge to lignite was higher than that of 1:1 and 0:1 ratios in terms of gene expressions, correlating with the coenzyme A pathway of the reduced acetate.

As shown in **Figure 3**, sugars were converted from glucose to pyruvate through the action of hydrolytic acidifying enzymes. In **Figure 4(a)**, pyruvate is reduced to acetic acid by pyruvate dehydrogenase (EC:1.2.4.1) in the absence of oxygen, and oxidized by dihydrolipoyl lysine-residue acetyltransferase (EC:2.3.1.12) and acetyl-CoA synthetase (EC:6.2.1.1) in the presence of oxygen, generating acetyl coenzyme A [50]. The differences between the three different ratios of residual sludge and lignite in on the glycolytic pathway were more pronounced (**Figure**

4(b).

Overall, there was a positive trend of correlation between sludge addition and gene abundance. Thus, the abundance of gene expression was relatively high in response groups with higher sludge content. Figure 4(c) illustrates several genes with higher abundance. There was a substantial difference in gene abundance within the glycolytic pathway, with genes in the 3:1 ratio being much more prevalent than those in the 1:1 and 0:1 ratios. In the reduced acetyl coenzyme A pathway, although gene abundance was high, the difference was not statistically significant, correlating with the results depicted in Figure 4(b). As a result, the system featuring a 3:1 ratio of residual sludge to lignite exhibited higher glycolytic metabolic activity. This heightened activity proved conducive, promoting acidification during anaerobic processes.

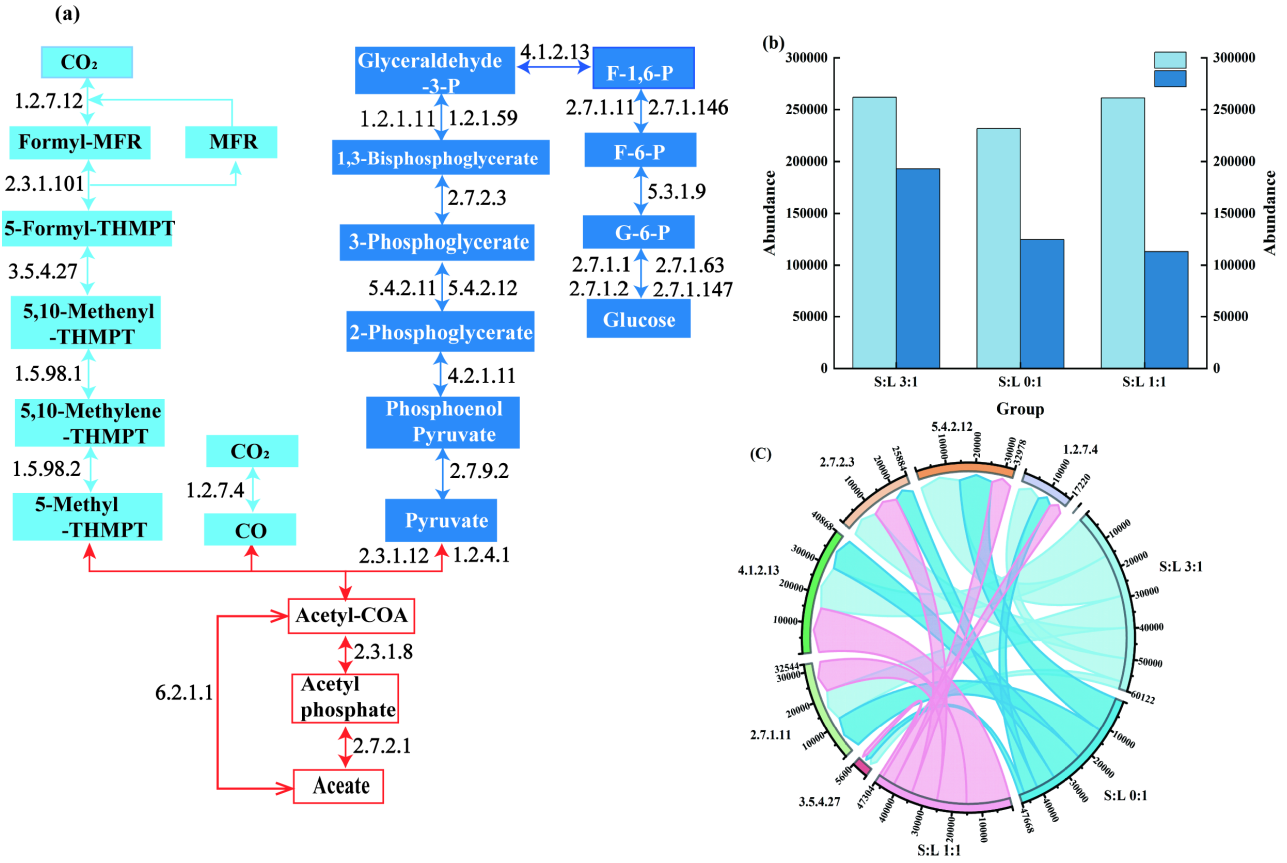

Figure 4. Comparative analysis of acetate metabolic pathways (a) Acetic acid synthesis pathway (b) Comparison of gene abundance of two pathways of acetic acid synthesis (c) Abundance of key enzymes.

3.3.3. Methanation Stages and Associated Enzyme Activities

Biomethane metabolism is associated with organic matter degradation and its transformation during anaerobic fermentation. Furthermore, it was crucial to clarify the metabolic pathways of the archaeal community. To evaluate the process of biomethane metabolism in three groups of the different ration systems, genes encoding key enzymes related to methane production were

recorded (Table 5). In order to further elucidate the effect of sludge addition on producer methane, the promotion mechanism was analyzed through the methanogenic metabolic pathway. The results of this analysis are presented in Figure 5.

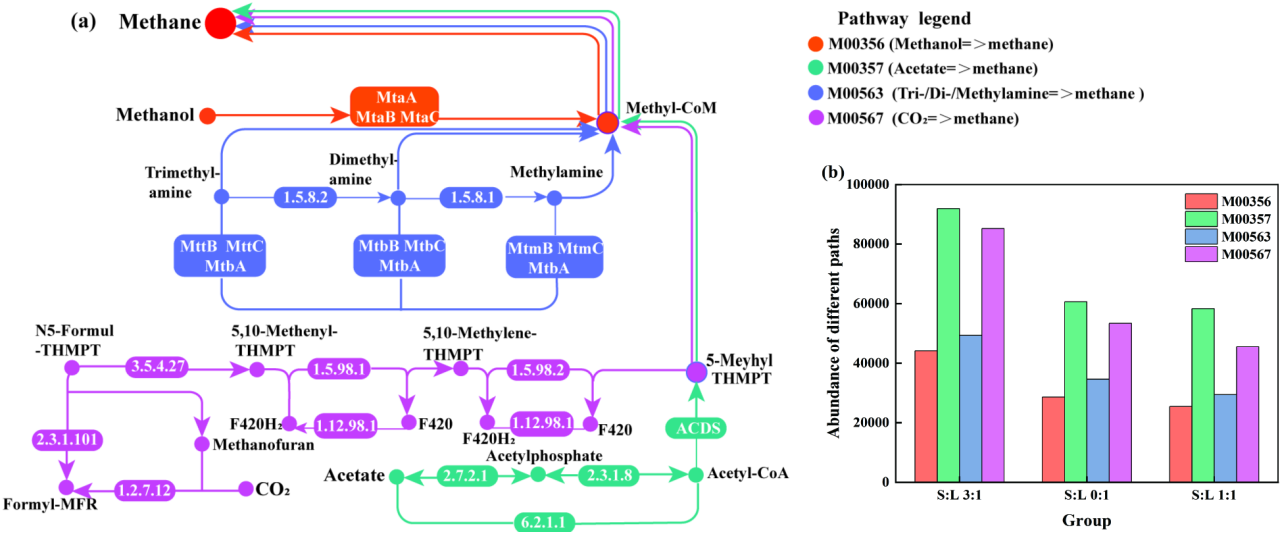

Figure 5. Methanogenesis pathways and the abundances of M00356, M00357, M00563 and M00567 from using the KEGG modules (a) Metabolic pathways (b) Abundance of different pathways.

Methane metabolism was the last stage of anaerobic fermentation and methanogenic archaea, played a major role in this stage. Furthermore, using the KEGG database to identify the substance metabolic pathways and methanogenic pathways in the anaerobic fermentation process, various types of metabolic pathways in the methanation pathway of three different ratios of residual sludge and lignite systems were analyzed (Figure 5 and Table 5). Figure 5(a) depicts the methanation pathway and the associated biological enzyme species in the methanation pathway. The methanogenic pathways were divided into four: M00356 (methanol methanation), M00357 (decarboxylation of acetic acid), M00563 (methanation of methylamine/d-dimethylamine/trimethylamine), and M00567 (reduction of CO_2).The proportion of acetic acid decarboxylation pathway (9.62% - 15.31%) was higher compared with the CO_2 reduction pathway (7.51% - 14.10%), methylamine/dim-ethylamine/t-rimethylamine methanation pathway (4.93% - 8.12%), and methanol methanation pathway (4.14% - 4.09%) for all three proportioned reaction systems. It was observed that in the process of anaerobic fermentation, the metabolism of acetic acid served as a substrate for heterotrophic microorganisms. The metabolic utilization of acetic acid as a substrate was found to be more competitive compared with other microorganisms during anaerobic fermentation. Meanwhile, archaea with CO_2 as the substrate were expressed in higher abundance in the system with a 3:1 sludge-to-lignite ratio, resulting in a higher proportion of the CO_2 reduction pathway in the methanation pathway in the 3:1 reaction system.

Table 5. Typical KEGG-based methane metabolic pathway-related genes and their abundance.

Pathway	EC number	K number	Abundance		
			0:1	1:1	3:1
M00567 (Methanogenesis, CO_2 → methane)	1.2.7.12	K00201	3782	3512	5902
		K00202	1802	1858	3070
		K00203	1306	1188	1906
		K00205	2776	2146	5260
		K11261	1940	1390	3588
		K11260	890	668	1544
		K00200	2448	2620	3698
	2.3.1.101	K00672	1818	1454	2766
	3.5.4.27	K01499	1788	1312	2500
	1.5.98.1	K00319	1276	946	2448
	1.5.98.2	K00320	3618	3780	2342
	2.7.2.1	K00925	3382	7432	8016
M00357 (Methanogenesis, acetate → methane)	2.3.1.8	K00625	2248	1692	4774
	2.3.1.169 (ACDS)	K00193	3326	2028	1884
	2.1.245 (ACDS)	K00194	1460	2030	3192
		K13788	362	2628	50
	6.2.1.1	K01895	17,756	17,852	18,538
M00356 (Methanogenesis, methanol → methane)	mtaA	K14080	2780	3074	3666
	2.1.1.90	K04480	2548	2872	3016
		K14081	1938	2170	2302
	mttC	K14084	1636	1858	2106
M00563 (Methanogenesis, methylamine/dimethylamine/trimethylamine → methane)	mtbB	K16178	1684	1876	2246
	mtbC	K16179	1546	1432	1980
	mtmB	K16176	2394	2420	2980
	mtmC	K16177	784	972	1078

As shown in Table 5, the key enzymes involved in the conversion of acetic acid during the methanation of acetic acid were acetate kinase (*ack*, EC: 2.7.2.1), phosphate acetyltransferase (*pta*, EC: 2.3.1.8), acetyl-CoA synthase (*acs*, EC:6.2.1.1), and acetyl-CoA synthase [51]. Acetic acid underwent two processes, the conversion of acetic acid to acetyl coenzyme A and the conversion of acetyl coenzyme A to 5-methyl-THMPT, before it is converted to methane. (**Figure 5(a)**). As seen from the data in Table 5, the gene abundance of the acs system was significantly higher than that of the ack-pta system. This indicated that the pathway involved in the acs system is the main pathway for the conversion of acetic acid to acetyl coenzyme A in the reaction. However, in the hydrogenotrophic (M00567) methanogenesis pathway, as shown in **Figure 5**, CO_2 is reduced to a series of

intermediates by a variety of enzymes to form Methyl-CoM, which ultimately catalyzes the reduction to CH_4. The differences in gene abundance in the acetic acid decarboxylation pathway among the three reaction systems were small (15% for 3:1, 10% for 0:1, and 9.6% for 1:1). However, in the CO_2 reduction pathway, the gene abundance of the sludge to lignite ration of 3:1 was 186.6% of that of 1:1, and 159.09% of that of 0:1, indicating that the enzyme activity in the CO_2 reduction pathway of the 3:1 reaction system exhibited higher sludge addition. In summary, the biomethanation pathway of anaerobic fermentation of sludge and lignite mainly uses acetic acid and CO_2 as substrates for fermentation. Additionally, combining the typical genes and their abundance data correlated with the methane metabolic pathway in **Figure 2(c)** and **Table 5**. *Methanosarcina, Methanothrix* and *Methanosarcina-les* are the main archaea in the methanization pathway. The 3:1 group of the three reaction systems with higher sludge incorporation performed excellently in the methanation pathway.

4. Conclusion

In the anaerobic fermentation of three groups involving residual sludge and lignite with different proportioning systems for biomethane production, the cumulative methane production in the 3:1 proportioning system surpassed that of 1:1 and 0:1 by 1.64 and 1.16 times, respectively. Macrogenomic test results indicated that the 3:1 reaction group exhibited enrichment of dominant bacterial genera, including *Bacteroidetes, Bacteroidales,* and *Sphaerochaeta.* This enrichment favoured the hydrolysis of a wide range of organic matter. Additionally, the 3:1 reaction group, exhibited enhanced enrichment of methanogenic archaea such as *Methanosarcina*, *Methanothrix and Methanosarcinales,* positively impacting the acetate decarboxylation and CO_2 reduction pathways compared with the 1:1 and 0:1 groups. These factors contributed to enhanced subsequent methanogenesis. Meanwhile, the relative gene abundance of functional metabolism enzymes such as acetate kinase, phosphate acetyltransferase, acetyl-CoA synthase, and acetate-CoA ligase in the metabolic pathways of hydrolytic acidification, acetic acid synthesis, and methanation was notably higher in the reaction group with the ratio system of 3:1. This increase enhanced the efficiency of the reaction system. Furthermore, the findings indicated that the addition of sludge played a crucial role in enhancing relevant enzyme activities during the anaerobic fermentation of lignite.

Acknowledgements

This work was supported by theNational Natural Science Foundation of China (42172199, 42102218); the Key project supported by National Natural Science Foundation of China (42230804); the Outstanding Youth Science Foundation of Henan Province (202300410168); Open Fund projects (Key Laboratory of Coalbed Methane Resources and Depository Processes, Ministry of Education (China University of Mining and Technology), 2022-03).

Data Availability

Data will be made available on request.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Melikoglu, M. (2018) Clean Coal Technologies: A Global to Local Review for Turkey. *Energy Strategy Reviews*, **22**, 313-319. https://doi.org/10.1016/j.esr.2018.10.011

[2] Chen, X., Huang, J., Yang, Q., Nielsen, C.P., Shi, D. and McElroy, M.B. (2018) Changing Carbon Content of Chinese Coal and Implications for Emissions of CO_2. *Journal of Cleaner Production*, **194**, 150-157. https://doi.org/10.1016/j.jclepro.2018.05.128

[3] Wang, A.K. and Qin, Y. (2010) Research Status and Progress of Experimental Study on Biogenic Coalbed Methane. *Coal Geology & Exploration*, **38**, 23-27.

[4] Bao, Y., Huang, H., He, D., Ju, Y. and Qi, Y. (2016) Microbial Enhancing Coal-Bed Methane Generation Potential, Constraints and Mechanism—A Mini-Review. *Journal of Natural Gas Science and Engineering*, **35**, 68-78. https://doi.org/10.1016/j.jngse.2016.08.035

[5] Chen, T., Zheng, H., Hamilton, S., Rodrigues, S., Golding, S.D. and Rudolph, V. (2017) Characterisation of Bioavailability of Surat Basin Walloon Coals for Biogenic Methane Production Using Environmental Microbial Consortia. *International Journal of Coal Geology*, **179**, 92-112. https://doi.org/10.1016/j.coal.2017.05.017

[6] Zhang, T., Liu, X. and He, Q. (2019) Research Progress on Municipal Sewage Sludge Disposal Technology and Resource Utilization. *Light Industry Technology*, **35**, 94-95.

[7] Wang, L., He, R. and Lei, H. (2022) Overview of the Current Status of Sludge Treatment and Disposal Technologies in Urban Wastewater Treatment Plants. W*ater Purification Technology*, **41**, 16-21, 69.

[8] Ma, Y., Wu, J. and Wang, P. (2024) Progress of Acid Production by Anaerobic Co-Fermentation of Residual Sludge under Low-Carbon Background. *Environmental Engineering*, **3**, 1-11.

[9] Gao, W. and Cheng, H. (2023) Research Progress of Sludge Treatment and Disposal Technology in China. *Chemical Minerals and Processing*, **52**, 71-79.

[10] Chang, C., Ming, L. and Mou, Y. (2022) Synergistic Effect of Food Waste and Sludge Anaerobic Fermentation for Methane Production. *China Environmental Science*, **42**, 1259-1266.

[11] Guo, H., Cheng, Y., Huang, Z., Urynowicz, M.A., Liang, W., Han, Z., *et al.* (2019) Factors Affecting Co-Degradation of Coal and Straw to Enhance Biogenic Coalbed Methane. *Fuel*, **244**, 240-246. https://doi.org/10.1016/j.fuel.2019.02.011

[12] Feng, X., Lu, L. and Xie, Y. (2018) A Preliminary Investigation of Microbial Degradation Conditions of Coal in Activated Sludge. *Journal of Tianjin Polytechnic University*, **34**, 47-50.

[13] Wang, A., Qin, Y. and Shao, P. (2015) Characteristics of Coal Particle Size on Lignite Biogas Generation. *China Coalbed Methane*, **12**, 3-6.

[14] Wei, X., Chen, Y. and Zhang, A. (2021) Study on the Effect of Coal Sample Particle Size on Gas Production, Oxygen Consumption and Pontaneous Combustion Propensity.

Coal and Chemical Industry, **44**, 99-102.

[15] Wang, B., Tai, C., Wu, L., Chen, L., Liu, J., Hu, B., *et al.* (2017) Methane Production from Lignite through the Combined Effects of Exogenous Aerobic and Anaerobic Microflora. *International Journal of Coal Geology*, **173**, 84-93. https://doi.org/10.1016/j.coal.2017.02.012

[16] Guo, H., Zhao, S., Dong, Z., Wang, Q., Xia, D., Jia, J., *et al.* (2020) Clean and Efficient Utilization of Coal Combined with Corn Straw by Synergistic Biodegradation. *Renewable Energy*, **161**, 701-711. https://doi.org/10.1016/j.renene.2020.07.023

[17] Wang, C., Feng, Z. and Wang, X. (2021) Effect of Functional Groups in Coal on the Depth of Adsorption Potential Well. *Adsorption Science & Technology*, **2021**, Article 3820762. https://doi.org/10.1155/2021/3820762

[18] Hill, V.R., Kahler, A.M., Jothikumar, N., Johnson, T.B., Hahn, D. and Cromeans, T.L. (2007) Multistate Evaluation of an Ultrafiltration-Based Procedure for Simultaneous Recovery of Enteric Microbes in 100-Liter Tap Water Samples. *Applied and Environmental Microbiology*, **73**, 6327-6327. https://doi.org/10.1128/aem.01863-07

[19] Chen, K., Deng, X., Wang, L., He, R., Yang, Y., Jiang, J., *et al.* (2021) Effects of Different Functional Strains on Key Metabolic Pathways of Methanogenesis in the Domestic Waste Fermentation Reactor. *Biomass and Bioenergy*, **146**, Article 105995. https://doi.org/10.1016/j.biombioe.2021.105995

[20] Venkiteshwaran, K., Milferstedt, K., Hamelin, J., Fujimoto, M., Johnson, M. and Zitomer, D.H. (2017) Correlating Methane Production to Microbiota in Anaerobic Digesters Fed Synthetic Wastewater. *Water Research*, **110**, 161-169. https://doi.org/10.1016/j.watres.2016.12.010

[21] Zhang, T., Shao, M. and Ye, L. (2011) 454 Pyrosequencing Reveals Bacterial Diversity of Activated Sludge from 14 Sewage Treatment Plants. *The ISME Journal*, **6**, 1137-1147. https://doi.org/10.1038/ismej.2011.188

[22] Chen, Y., Liu, G., Fan, Q., Wang, J., Qi, L. and Wang, H. (2015) Effect of Carbon Removal and Denitrification and Change of Bacterial Community Structure in A/O System under Different Dissolved Oxygen Conditions. *Environmental Science*, **7**, 2610-2616.

[23] Khesali Aghtaei, H., Püttker, S., Maus, I., Heyer, R., Huang, L., Sczyrba, A., *et al.* (2022) Adaptation of a Microbial Community to Demand-Oriented Biological Methanation. *Biotechnology for Biofuels and Bioproducts*, **15**, Article No. 125. https://doi.org/10.1186/s13068-022-02207-w

[24] Fu, H., Yan, D., Su, X., Wang, J., Li, Q., Li, X., *et al.* (2022) Biodegradation of Early Thermogenic Gas and Generation of Secondary Microbial Gas in the Tieliekedong Region of the Northern Tarim Basin, NW China. *International Journal of Coal Geology*, **261**, Article 104075. https://doi.org/10.1016/j.coal.2022.104075

[25] Li, Y., Fu, H., Yan, D., Su, X., Wang, X., Zhao, W., *et al.* (2022) Effects of Simulated Surface Freshwater Environment on in Situ Microorganisms and Their Methanogenesis after Tectonic Uplift of a Deep Coal Seam. *International Journal of Coal Geology*, **257**, Article 104014. https://doi.org/10.1016/j.coal.2022.104014

[26] Won, M., Oyama, L.B., Courtney, S.J., Creevey, C.J. and Huws, S.A. (2020) Can Rumen Bacteria Communicate to Each Other? *Microbiome*, **8**, Article No. 23. https://doi.org/10.1186/s40168-020-00796-y

[27] Regueiro, L., Lema, J.M. and Carballa, M. (2015) Key Microbial Communities Steering the Functioning of Anaerobic Digesters during Hydraulic and Organic Overloading Shocks. *Bioresource Technology*, **197**, 208-216.

https://doi.org/10.1016/j.biortech.2015.08.076

[28] Ziganshina, E.E., Belostotskiy, D.E., Ilinskaya, O.N., Boulygina, E.A., Grigoryeva, T.V. and Ziganshin, A.M. (2015) Effect of the Organic Loading Rate Increase and the Presence of Zeolite on Microbial Community Composition and Process Stability during Anaerobic Digestion of Chicken Wastes. *Microbial Ecology*, **70**, 948-960. https://doi.org/10.1007/s00248-015-0635-2

[29] Wang, C., Wei, S., Jin, M., Liu, B., Yue, M. and Wang, Y. (2022) Integrated Microbiomic and Metabolomic Dynamics of Fermented Corn and Soybean By-Product Mixed Substrate. *Frontiers in Nutrition*, **9**, Article 831243. https://doi.org/10.3389/fnut.2022.831243

[30] Liu, Y. and Whitman, W.B. (2008) Metabolic, Phylogenetic, and Ecological Diversity of the Methanogenic Archaea. *Annals of the New York Academy of Sciences*, **1125**, 171-189. https://doi.org/10.1196/annals.1419.019

[31] Lin, R., Cheng, J., Zhang, J., Zhou, J., Cen, K. and Murphy, J.D. (2017) Boosting Biomethane Yield and Production Rate with Graphene: The Potential of Direct Interspecies Electron Transfer in Anaerobic Digestion. *Bioresource Technology*, **239**, 345-352. https://doi.org/10.1016/j.biortech.2017.05.017

[32] Xu, R., Xu, S., Florentino, A.P., Zhang, L., Yang, Z. and Liu, Y. (2019) Enhancing Blackwater Methane Production by Enriching Hydrogenotrophic Methanogens through Hydrogen Supplementation. *Bioresource Technology*, **278**, 481-485. https://doi.org/10.1016/j.biortech.2019.01.014

[33] Zhao, Z., Wang, J., Li, Y., Zhu, T., Yu, Q., Wang, T., *et al.* (2020) Why Do Dieters Like Drinking: Metagenomic Analysis for Methane and Energy Metabolism during Anaerobic Digestion with Ethanol. *Water Research*, **171**, Article 115425. https://doi.org/10.1016/j.watres.2019.115425

[34] Huws, S.A., Edwards, J.E., Lin, W., Rubino, F., Alston, M., Swarbreck, D., *et al.* (2021) Microbiomes Attached to Fresh Perennial Ryegrass Are Temporally Resilient and Adapt to Changing Ecological Niches. *Microbiome*, **9**, Article No. 143. https://doi.org/10.1186/s40168-021-01087-w

[35] Hinsu, A.T., Tulsani, N.J., Panchal, K.J., Pandit, R.J., Jyotsana, B., Dafale, N.A., *et al.* (2021) Characterizing Rumen Microbiota and CAZyme Profile of Indian Dromedary Camel (*Camelus dromedarius*) in Response to Different Roughages. *Scientific Reports*, **11**, Article No. 9400. https://doi.org/10.1038/s41598-021-88943-9

[36] Vanwonterghem, I., Jensen, P.D., Rabaey, K. and Tyson, G.W. (2016) Genome-Centric Resolution of Microbial Diversity, Metabolism and Interactions in Anaerobic Digestion. *Environmental Microbiology*, **18**, 3144-3158. https://doi.org/10.1111/1462-2920.13382

[37] Ping, Q., Zheng, M., Dai, X. and Li, Y. (2020) Metagenomic Characterization of the Enhanced Performance of Anaerobic Fermentation of Waste Activated Sludge with CaO_2 Addition at Ambient Temperature: Fatty Acid Biosynthesis Metabolic Pathway and CAZymes. *Water Research*, **170**, Article 115309. https://doi.org/10.1016/j.watres.2019.115309

[38] Moraïs, S. and Mizrahi, I. (2019) Islands in the Stream: From Individual to Communal Fiber Degradation in the Rumen Ecosystem. *FEMS Microbiology Reviews*, **43**, 362-379. https://doi.org/10.1093/femsre/fuz007

[39] Wintsche, B., Jehmlich, N., Popp, D., Harms, H. and Kleinsteuber, S. (2018) Metabolic Adaptation of Methanogens in Anaerobic Digesters upon Trace Element Limitation. *Frontiers in Microbiology*, **9**, Article No. 405. https://doi.org/10.3389/fmicb.2018.00405

[40] Long, C., Qi, X. and Venema, K. (2022) Chemical and Nutritional Characteristics, and Microbial Degradation of Rapeseed Meal Recalcitrant Carbohydrates: A Review. *Frontiers in Nutrition*, **9**, Article 948302. https://doi.org/10.3389/fnut.2022.948302

[41] White, B.A., Lamed, R., Bayer, E.A. and Flint, H.J. (2014) Biomass Utilization by Gut Microbiomes. *Annual Review of Microbiology*, **68**, 279-296. https://doi.org/10.1146/annurev-micro-092412-155618

[42] Jiang, X., Yan, Y., Feng, L., Wang, F., Guo, Y., Zhang, X., *et al.* (2021) Bisphenol A Alters Volatile Fatty Acids Accumulation during Sludge Anaerobic Fermentation by Affecting Amino Acid Metabolism, Material Transport and Carbohydrate-Active Enzymes. *Bioresource Technology*, **323**, Article 124588. https://doi.org/10.1016/j.biortech.2020.124588

[43] Stam, M.R., Danchin, E.G.J., Rancurel, C., Coutinho, P.M. and Henrissat, B. (2006) Dividing the Large Glycoside Hydrolase Family 13 into Subfamilies: Towards Improved Functional Annotations of Amylase-Related Proteins. *Protein Engineering Design and Selection*, **19**, 555-562. https://doi.org/10.1093/protein/gzl044

[44] Zhang, M., Guo, H., Xia, D., Dong, Z., Liu, X., Zhao, W., *et al.* (2022) Metagenomic Insight of Corn Straw Conditioning on Substrates Metabolism during Coal Anaerobic Fermentation. *Science of the Total Environment*, **808**, Article 152220. https://doi.org/10.1016/j.scitotenv.2021.152220

[45] Yang, G., Xu, C., Varjani, S., Zhou, Y., WC Wong, J. and Duan, G. (2022) Metagenomic Insights into Improving Mechanisms of Fe^0 Nanoparticles on Volatile Fatty Acids Production from Potato Peel Waste Anaerobic Fermentation. *Bioresource Technology*, **361**, Article 127703. https://doi.org/10.1016/j.biortech.2022.127703

[46] Chen, Y., Liu, H., Zheng, X., Wang, X. and Wu, J. (2017) New Method for Enhancement of Bioenergy Production from Municipal Organic Wastes via Regulation of Anaerobic Fermentation Process. *Applied Energy*, **196**, 190-198. https://doi.org/10.1016/j.apenergy.2017.01.100

[47] Cruz Ramos, H., Hoffmann, T., Marino, M., Nedjari, H., Presecan-Siedel, E., Dreesen, O., *et al.* (2000) Fermentative Metabolism of *Bacillus subtilis*: Physiology and Regulation of Gene Expression. *Journal of Bacteriology*, **182**, 3072-3080. https://doi.org/10.1128/jb.182.11.3072-3080.2000

[48] Wang, D., Han, Y., Han, H., Li, K., Xu, C. and Zhuang, H. (2018) New Insights into Enhanced Anaerobic Degradation of Fischer-Tropsch Wastewater with the Assistance of Magnetite. *Bioresource Technology*, **257**, 147-156. https://doi.org/10.1016/j.biortech.2018.02.084

[49] Chen, D., Zuo, X., Li, J., Wang, X. and Liu, J. (2020) Carbon Migration and Metagenomic Characteristics during Anaerobic Digestion of Rice Straw. *Biotechnology for Biofuels*, **13**, Article No. 130. https://doi.org/10.1186/s13068-020-01770-4

[50] Du, J., Yin, Q., Gu, M. and Wu, G. (2021) New Insights into the Effect of Ethanol and Volatile Fatty Acids Proportions on Methanogenic Activities and Pathways. *Environmental Research*, **194**, Article 110644. https://doi.org/10.1016/j.envres.2020.110644

[51] Li, Y., Zhao, J. and Zhang, Z. (2021) Implementing Metatranscriptomics to Unveil the Mechanism of Bioaugmentation Adopted in a Continuous Anaerobic Process Treating Cow Manure. *Bioresource Technology*, **330**, Article 124962. https://doi.org/10.1016/j.biortech.2021.124962